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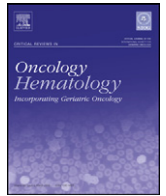
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Resistance mechanisms after tyrosine kinase inhibitors afatinib and crizotinib in non-small cell lung cancer, a review of the literature



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ABSTRACT

Targeted treatment of advanced non-small cell lung cancer patients with afatinib in *EGFR* mutation or crizotinib in *ALK* break positive patients results in profound tumor responses but inevitably induces resistance. In this review we present currently known resistance mechanisms for afatinib and crizotinib two recently approved drugs.

Resistance mechanisms identified for afatinib include *c-MET* amplification and the V843I *EGFR* mutation. Expression of FGFR1, increased IL6R/JAK/STAT signaling, enhanced interference with aerobic glycolysis and autophagy are associated with resistance to afatinib. Most common resistance

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mechanisms for ALK break positive cases are gatekeeper mutations in the ALK gene. Also activation of the EGFR pathway, KRAS mutations, the autophagy pathway and epithelial mesenchymal transition (EMT), have been associated with resistance. Many of the proposed resistance mechanisms need to be functionally studied to proof a causative relationship with resistance.

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Table 1
Overview of drugs used as EGFR-TKI or ALKi and their most important targets.

Drugs	Targets
Afatinib	EGFR, HER2, HER3, HER4
Dacomitinib	EGFR, HER2, HER4
Erlotinib	EGFR
Gefitinib	EGFR
Osimertinib	EGFR
Rociletinib	EGFR, IGF1R
Alectinib	ALK, RET
Brigatinib	ALK, EGFR
Ceritinib	ALK, ROS1, IFG1R
Crizotinib	MET, ALK, ROS1
Lorlatinib	ALK, ROS1

1. Introduction

Patients with an activating mutation in the epidermal growth factor receptor (EGFR) gene, e.g. deletion in exon 19 or L858R mutation, occur in about 8% of non-small cell lung cancer (NSCLC) patients (Kerner et al., 2013). In more than 70% of patients treatment is successful with EGFR-tyrosine kinase inhibitors (TKIs). However, resistance following treatment with TKIs eventually emerge in all of these patients. Most clinical data have been gathered with the first generation reversible EGFR inhibitors gefitinib and erlotinib (Table 1).

Second-generation EGFR-TKIs, e.g. afatinib and dacomitinib, form covalent irreversible bonds with the target that may increase their effectiveness through a more effective inhibition of EGFR signaling. At this moment also 3rd generation TKIs are under investigation in phase III studies, e.g. osimertinib and rociletinib, for patients with a EGFR T790M mutation, because these TKI bind more specific to this altered tyrosine kinase binding pocket (Jänne et al., 2015; Sequist et al., 2015). Around five percent of NSCLC patients showed to have a chromosomal inversion involving the Echinoderm Microtubule-associated protein-Like 4 (EML4) gene and the Anaplastic Lymphoma Kinase (ALK) gene. These patients can be treated with ALK inhibitors (ALKi), e.g. crizotinib and ceritinib (Table 1) (Kwak et al., 2010). At this moment alectinib only has only been approved in Japan. Ceritinib and alectinib bind more specific with the tyrosine kinase of pocket of the ALK protein due to covalent binding and the fact that these are smaller molecules than crizotinib. The median time to progression is 8–12 months for all TKIs when administered as first-line treatment, which is better than chemotherapy (Mok et al., 2009; Rosell et al., 2012; Shaw et al., 2013; Sequist et al., 2013). The median progression free survival (PFS) is comparable to first line treatment when these TKIs are used as second-line treatment following chemotherapy (Mok et al., 2013). In subsequent treatment lines however survival is not different from chemotherapy.

Many genetic aberrations have been described as possible mechanisms for resistance towards TKIs (Rosell et al., 2013). In this review we will focus on resistance mechanisms, found in tumor biopsies of NSCLC patients, xenograft models and cell lines that emerge after treatment with or exposure to EGFR-TKI afatinib (Boehringer Ingelheim GmbH, Germany) and the ALKi crizotinib (Pfizer Inc., City of New York, NY). Both afatinib and crizotinib

have only recently been approved for treatment and knowledge on known and possible resistance mechanisms is essential for clinicians.

2. Resistance after EGFR tyrosine kinase inhibitors

Afatinib, erlotinib and gefitinib are registered as first line treatment for patients with advanced NSCLC with activating EGFR mutations. Afatinib is the only proven drug showing an increased overall survival as first line treatment in patients with an exon 19 deletion as compared to chemotherapy, i.e. 31.7 (95% confidence interval (CI) of 28.1–35.1) vs. 20.7 (95% CI of 16.3–25.6) months, respectively (Miller et al., 2012; Yang et al., 2015). For patients with a L858R mutation OS however is comparable between afatinib and chemotherapy treatment (OS 22.1 months for afatinib; 95% CI of 19.6–25.4 vs. 26.9 months for chemotherapy; 95% CI of 23.2–31.7) (Yang et al., 2015). An overview of currently known resistance mechanisms is given below. It consists of a summary of findings with first generation TKIs followed by a more extensive overview of resistance mechanisms associated with afatinib only.

2.1. Erlotinib and gefitinib resistance in NSCLC patients, cell lines and mouse models

The T790M mutation is the most common observed resistance mechanism (30–83%) in patients treated with first generation EGFR-TKIs (Arcila et al., 2011; Chen et al., 2009; Hata et al., 2013; Su et al., 2012; Yu et al., 2013; Lee et al., 2014). The resistant tumor clones may originate from a minor pre-existing T790M mutation positive tumor cell subclone or present as a *de novo* mutation (Nguyen et al., 2009). Mutation screening of >2700 lung pre-treatment cancer samples with an EGFR mutation revealed a T790M mutation in only 20 cases. This may indicate that without TKI selective pressure the frequency of the T790M is very low (Yu et al., 2014a). The T790M mutation has also been described as a germ line variant co-segregating in a family with the development of lung adenocarcinoma (Oxnard et al., 2012; Yu et al., 2014b). A similar co-segregation with lung cancer was seen in a family carrying a germ line V843I and a family with a P848L EGFR mutation (Matsushima et al., 2014; Ohtsuka et al., 2011; Prim et al., 2014). It is therefore most likely that resistant mutations are already present in minor clones of the tumor. In a clinical subgroup analysis of EGFR mutant patients in the EORTC trial pretreatment T790M mutation was associated with a decrease in PFS. Bcl-2 interacting mediator of cell death (BCL2L11, also known as BIM) mRNA expression was associated with longer PFS and OS in the same patients treated with erlotinib (Costa et al., 2014). Mice, with lung epithelial cell specific overexpression of activated EGFR that developed lung tumors and who were treated with erlotinib, revealed a T790M mutation in 5/17 and in 5/17 different mice a MET gene amplification (Politi et al., 2010). Indicating that both T790M and MET gene amplification are potential resistance mechanisms to erlotinib. HER2 amplifications and mutations were observed in lung cancer biopsies in respectively 10% and 2% of tumors with acquired resistance to erlotinib and gefitinib, but only in 1% of untreated tumors (Mazières et al., 2013). Therefore, in addition HER2 may be

responsible for resistance emerging under pressure of treatment especially with erlotinib. Phosphorylation of Src family kinase (SFK) was identified in tumor samples of patients treated with first generation TKIs (Yoshida et al., 2014). Additional resistance mechanisms such as expression of HGF, the ligand for *MET* or increased activation of the AXL pathway, up-regulation of NF- κ B, GAS6, ADAM17, NOTCH1, p53, Wnt and mTor were reported in studies focusing on tumor samples and cell lines (Rosell et al., 2013; Zhang et al., 2012; Huang et al., 2011; Baumgart et al., 2010; Fong et al., 2013; Bivona et al., 2011; Chen et al., 2013a).

2.2. Afatinib resistance in NSCLC patients

In Asian patients the presence of T790M mutations in re-biopsies obtained before second line treatment with afatinib treatment had no prognostic or predictive role (Sun et al., 2013a). This observation was confirmed in a study where a combination of afatinib and cetuximab (a monoclonal antibody against EGFR) showed tumor responses irrespective of the presence of T790M (Table 2) (Janjigian et al., 2014). Thus questioning as to whether a T790M mutation is important as a resistance mechanism upon treatment with afatinib. Afatinib is equally potent against wild-type EGFR and EGFR T790M and therefore side effects resulting from inhibiting wild-type EGFR (rash and diarrhea) prevents the suppression of T790M tumor cells at clinically achievable doses. In addition, patients treated with afatinib have more side effects, e.g. diarrhea and skin rash, than observed with erlotinib or gefitinib treated patients (Takeda et al., 2015). Third generation EGFR TKIs such as rociletinib, osimertinib, or EGF816, target mutant EGFR T790M and largely spares wild type EGFR, thereby decreasing toxicity and therefore permit clinical doses that fully suppress T790M. That explains their efficacy in T790M positive tumors.

2.3. Afatinib resistance in cell lines, xenografts and mouse models

2.3.1. Gatekeeper mutations

Multiple cell lines have been used in the search for responsiveness and resistance mechanisms to afatinib. A summary of the endogenous and exogenous alterations in relation to TKI responsiveness is given in Table 3.

Cell lines carrying the activating exon 19 delE746_A750 or L858R mutations are sensitive to afatinib (Matsushima et al., 2014). PLA1 cells with the activating L858R in combination with the resistant V843I mutation are more resistant to afatinib than H1975 cells with a L858R in combination with the T790M resistant mutation. This difference in sensitivity can be explained by conformational change of the predicted TKI binding site as a consequence of the V843I and the T790M mutations (Matsushima et al., 2014). Both EGFR mutations are referred to as gatekeeper mutations, of which V843I appears to be a more resistant variant than T790M. In another study afatinib inhibits the growth of the H1975 cell line (containing the L858R/T790M mutation) in vitro as well as in the xenograft model (Li et al., 2008). In comparison to EGFR-TKIs, EGFR siRNAs were much more effective in growth inhibition of lung cancer cells (Chen et al., 2012). This enhanced efficiency occurred especially in T790M mutation positive lung cancer cell lines (Chen et al., 2013b). This indicates that treatment with EGFR-TKIs only partly block the EGFR signaling pathway, in which especially T790M seems to be important as a resistance mechanism in cell lines.

Resistance upon exposure to afatinib in PC9 cells seems to be irreversible indicating that the resistance has been caused by a genetic change (Kim et al., 2012a). The majority of the resistant subclones remained dependent on EGFR signaling as shown by siRNA treatment. These EGFR dependent PC9 clones all gained a T790M mutation and cell viability was dependent of the T790M allele dosage (Kim et al., 2012a). However, in the xenograft

model no difference in tumor response was observed at maximum dose for afatinib between different T790M allele dosages. Other known resistance mechanisms such as amplification of *MET*, deletion of phosphatase and tensin homolog (*PTEN*) and epithelial-mesenchymal transition (EMT), were not observed in the afatinib resistant, EGFR independent PC9 subclones.

In a mouse model, overexpression of L858R mutant EGFR in epithelial lung cells resulted in the development of lung cancer (Politi et al., 2010). Upon treatment of these mice with erlotinib, resistant tumor clones gained a T790M mutation, which showed a modest response to afatinib monotherapy. Combining afatinib with cetuximab greatly enhanced the response with complete responses in most cases (7/8) (Regales et al., 2009). This indicates that adding cetuximab to afatinib induces a higher response rate in T790M positive tumor cells. Another study suggested that this effect was related to the heterodimerization of tyrosine-phosphorylated EGFR with HER2, to which afatinib is a known inhibitor as well (Takezawa et al., 2012). The differences observed between the cell line and mouse models in comparison to the above-discussed findings in patient samples are caused by differences in clinical achievable afatinib doses. Such afatinib doses are too low to suppress T790M mutated tumors.

2.3.2. HER2 gene expression and receptor involvement in resistance

Knockdown of *HER2* in PC9, HCC827 and H3255 cells increased sensitivity to afatinib (Takezawa et al., 2012). This is consistent with the improved response rate observed in patients treated with afatinib and cetuximab as compared to erlotinib and cetuximab or afatinib alone. Erlotinib-resistant PC9 and HCC827 derived xenograft tumors are characterized by enhanced levels of phosphorylated HER2. Proliferation of these HER2 positive tumor cells in nude mice could be inhibited by afatinib or cetuximab, with the strongest inhibition of HER2 being observed for treatment with afatinib/cetuximab (Takezawa et al., 2012). Thus *HER2* is a mechanism of resistance induced upon treatment with erlotinib and gefitinib. For afatinib however, a known pan-HER inhibitor, this mechanism is not relevant.

2.3.3. MET gene expression and receptor involvement in resistance

Amplification and/or mutations of *MET* have been identified in two of the lung cancer cell lines and may represent a possible escape mechanism to afatinib. Treatment of lung cancer cell lines harboring activating EGFR mutations with crizotinib (a known MET and ALK inhibitor) showed a marked inhibitory effect on cell growth of *MET* amplification positive cell lines, indicating that these cells were addicted to the MET pathway. The MET addiction was confirmed by increased levels of apoptosis upon MET inhibition in *MET* amplification positive cell lines, EBC-1 and H1993 (Chen et al., 2013b; Tanizaki et al., 2011). EGFR mutation positive cell lines with mutant or wild type *MET* were not or less sensitive to crizotinib and/or MET siRNAs, indicating that a *MET* mutation is not important in resistance in these cells (Chen et al., 2013b; Tanizaki et al., 2011). Combination of MET siRNAs with either EGFR-TKIs, including afatinib, or EGFR siRNAs, revealed decreased proliferation as compared to EGFR siRNA alone (Chen et al., 2013b). In the T790M positive H1975 cell line, the synergistic effect was modest, also when the MET siRNA was combined with a siRNA directed specifically against T790M. Concomitant treatment of the cell lines with a MET inhibitor (SU11274) caused increased sensitivity for gefitinib, erlotinib, afatinib or cetuximab, especially for the H1975 cells. The combined effect was most pronounced for the combination of SU11274 and afatinib (Chen et al., 2009). In mice models, EGFR knock down in *MET* amplification positive tumors increased tumor cell death upon treatment with MET inhibitors. Mutations in *MET*

Table 2

Overview of re-biopsy studies in patients treated with afatinib.

Re-biopsy study	N	Mutation analysis	Treatment	mutations	PFS (mo)	reference
Sun et al. (2013)	70	direct sequencing and sequencing using a peptide nucleic acid	afatinib	T790M+ T790M–	14.7 14.1	Sun et al. (2013a)
Janjigian et al. (2014)	126	NA/different methods	afatinib/cetuximab	T790M+ T790M–	4.6 4.8	Janjigian et al. (2014)

Table 3Overview of IC₅₀ values in cell lines used in research on EGFR-TKI resistance.

Cell line	Endogenous EGFR/ induced alterations		Erlotinib (uM)	Gefitinib (uM)	Afatinib (uM)	Dacomitinib (uM)	Crizotinib (METi) (uM)	ref
	EGFR	other						
A549	wt		>100	>100	0.01	0.003	646	Matsushima et al. (2014), Tanizaki et al. (2011)
H292	wt		>10	0.06				Chen et al. (2013b)
H358	wt	KRAS codon 12	25	>10				Chen et al. (2009, 2013b)
H1299	wt						642	Tanizaki et al. (2011)
EBC1	wt	MET amp					5	Tanizaki et al. (2011)
H596	wt	MET exon 14del					752	Tanizaki et al. (2011)
H1437	wt	MET exon 14del					1284	Tanizaki et al. (2011)
H1993	wt	MET amp					10	Tanizaki et al. (2011)
H2122	wt	MET N375S					472	Tanizaki et al. (2011)
HCC827	exon 19del		<0.1	<0.1	0.01		767	Chen et al., (2009, 2012, 2013b), Tanizaki et al. (2011)
H1650	exon 19del		10–100	10–100	1–10			Chen et al. (2009, 2012)
PC9	exon 19del		0.03	0.02	<0.01		787	Takezawa et al. (2012), Tanizaki et al. (2011)
PC9-GR	EGFR T790M ↑		3441	100,000	312			Yoshida et al. (2014), Kim et al. (2012a), Lee et al. (2015), Coco et al. (2015)
PC9-BR		pHER2 ↓ pHER3 ↓ pFGFR1 ↑			0.02			Azuma et al. (2014)
H3255	L858R		0.04	0.04	0.0007			Takezawa et al. (2012)
H1975	L858R/T790M		100	100	1	10		Chen et al. (2009, 2012), Matsushima et al. (2014), Takezawa et al. (2012)
H1975-BR		EMT			29			Lee et al. (2015)
PLA1	L858R/V842I		>100	>100	1–10	>10		Matsushima et al. (2014)

wt: wild type; amp: amplification; GR: gefitinib resistant; BR: afatinib (BIBW2992) resistant.

did not affect sensitivity in mice models (Chen et al., 2009, 2013b; Tanizaki et al., 2011; Smolen et al., 2006). Thus, none of the studies support an effect of mutations or deletions of the *MET* gene towards TKI resistance, whereas amplification of *MET* does confer resistance to afatinib.

2.3.4. *STAT3* gene expression and receptor involvement in resistance

Two cell lines carrying the T790M *EGFR* mutation were used to explore the role of *STAT3* activation in the TKI-treatment induced resistance cells (Kim et al., 2012b). Afatinib treatment induced autocrine signaling of the JAK/STAT pathway via secretion of IL-6 in H1975 and PC9-gefitinib resistant (GR) cells. Blocking of the IL-6 receptor pathway (IL-6R) combined with afatinib treatment showed a more pronounced growth inhibition than afatinib alone. Vice versa, activation of the IL-6R signaling decreased sensitivity to afatinib. Moreover, an acquired resistance to afatinib was observed in PC9-GR xenograft mice that showed increased *STAT3* protein levels (Kim et al., 2012b). This indicates that upregulation of the *STAT3* pathway may contribute to afatinib resistance.

2.3.5. *FGFR1*

The PC9-BIBW9292 (afatinib) resistant cell line (BR), showed decreased mRNA expression levels of *HER2* and *HER3* compared

to the parental PC9 cells, whereas mRNA levels of *FGFR1* and its ligand *FGF2* were increased. Combining afatinib treatment with *FGFR1*-siRNAs or *FGFR1*-TKI inhibited cell growth of PC9-BR cells and induced a marked decrease in phosphorylation of AKT and ERK phosphorylation (Azuma et al., 2014). Thus, this afatinib resistant cell line was addicted to *FGFR1*-induced survival signals.

2.3.6. *SRC* family kinase (SFK)

In a tyrosine phospho-proteomic study in PC9-GR cells expression of many TKs was enhanced as compared to the wild type cells (Yoshida et al., 2014). Another study on SFK used a combination of dasatinib, a synthetic small molecule inhibitor of *SRC*-family protein-tyrosine kinases, and afatinib. This combination overcame the T790M mediated resistance of PC9-GR cells (IC₅₀ of 36uM as compared to 312uM for afatinib alone) (Huang et al., 2011). This result was confirmed in a xenograft model of nude mice injected with PC9-GR cells. These data indicate that SFK may present another mechanism to *EGFR*-TKI resistance.

2.3.7. *Heat shock protein 90* (HSP90)

There are no studies in which the effect of HSP90 inhibitors has been tested on afatinib resistant cell lines. However, in cell lines resistant to erlotinib the combination of afatinib and the HSP90 inhibitor ganetespib induced destabilization of *EGFR*, *MET*, p-*STAT3*

and p-AKT proteins gaining more cell death than afatinib or ganetespib alone (Smith et al., 2015). Ganetespib also induced a clear effect in CL-387,785 (pan-HER inhibitor) resistant clones or clones with an *ERBB2* exon 20 mutation (Shimamura et al., 2008, 2012). This means that although not studied for afatinib yet, HSP90 could play a role in treating resistance to a pan-HER inhibitor.

2.3.8. Autophagy

Combined treatment of T790M positive PC9 and H1975 cells with afatinib and suberoylanilide hydroxamic acid (SAHA), a potent reversible histone acetyltransferases and histone deacetylases (HDAC) inhibitor, could overcome the EGFR-TKI resistance through activation of the apoptotic pathway. The enhanced apoptosis was shown to be dependent on the autophagy pathway (Lee et al., 2015). These results were supported by *in vivo* data in xenograft mouse models.

2.3.9. Upregulation of other pathways

A genome wide screen at copy number, gene expression and protein levels in parental and afatinib resistant subclones revealed amongst others activation of AKT in H1975 cells (Coco et al., 2015). As activation of AKT has been associated with epithelial-mesenchymal transition (EMT) in prostate and breast cancer (Liu et al., 2014; Yang et al., 2014), EMT characteristics were also studied in the H1975 resistant subclones. This revealed loss of E-cadherin, decrease in cytokeratin levels, and increased expression level of mesenchymal markers such as vimentin. Based on these findings EMT was proposed as a possible mechanism of resistance (Coco et al., 2015). Another mode of action of inhibition of AKT may be an effect on the glycolytic pathway. Inhibition of the glycolysis by 2-deoxy-D-glucose (2DG) in H1975 and PC9-GR cells potentiated the sensitivity to afatinib. Two modes of action have been proposed. First, afatinib inhibits the PI3K/AKT pathway and prevents AKT from switching off the glycolytic pathway providing growth-enhancing signals to the tumor cells. Alternatively, the emergence of resistance towards afatinib lowers the AKT inhibition and switches on the glycolytic pathway (Kim et al., 2013a; Takezawa et al., 2010). Both mechanisms depend on the 2DG pathway, which explains the increased sensitivity towards afatinib upon inhibition of the 2DG pathway. Whether NF- κ B, GAS6, ADAM17, NOTCH1, Wnt, mTOR and p53, found to be associated with resistance to first generation TKIs (Rosell et al., 2013; Zhang et al., 2012; Huang et al., 2011; Baumgart et al., 2010; Fong et al., 2013; Bivona et al., 2011), play a role in resistance to afatinib is not obvious, as these mechanisms have not been studied yet.

3. Resistance after ALK tyrosine kinase inhibitors

Crizotinib is used as treatment in ALK translocation-positive patients. Besides ALK, crizotinib also targets MET and ROS1, although it has not been registered for these aberrations yet. Tumors of patients treated with crizotinib become insensitive to this drug after a median of 12 months (range: 1–34) (Shaw et al., 2013; Sequist et al., 2013). An overview of the currently proposed and known mechanisms to crizotinib based on patient samples and on *in vitro* data from cell line studies is discussed below.

3.1. ALK inhibitor resistance in NSCLC patients

Analysis of crizotinib resistant tumor clones demonstrated new ALK gene mutations in 17 out of 53 patients, e.g. L1151Tins, L1152R, C1156Y, L1196M, S1206Y, G1269A (Table 4) (Sasaki et al., 2011; Sang et al., 2013; Katayama et al., 2012; Kim et al., 2013b; Huang et al., 2013; Choi et al., 2010; Doebele et al., 2012). Most of these mutations affected the P-loop, β -sheet or α -helix of the ALK protein in the so-called gatekeeper area. Based on the crystal structure of

wild type and mutant (L1152R, G1202R, S1206Y and C1156Y) ALK protein, it was predicted that mutations close to the gatekeeper area resulted in a decreased interaction of crizotinib with the ALK receptor. *In vivo* experiments confirmed the accurateness of these predictions by increased resistance to crizotinib (Sun and Ji, 2012; Sun et al., 2013b). Thus these gatekeeper mutations prevent binding of crizotinib to the kinase domain of the ALK protein and due to this the binding of crizotinib becomes ineffective.

In addition to these gatekeeper mutations, ALK copy number gain has been found in almost 8% of resistant patients and EGFR or KRAS mutations have also been observed in almost 8% of the resistant tumor clones (Katayama et al., 2012; Kim et al., 2013b; Doebele et al., 2012; Jiang et al., 2013). Unusually, EGFR activating mutations were observed in 3/50 EML4-ALK positive, treatment naïve patient samples in only one study. This indicates that ALK breaks may co-exist with EGFR mutations (Sasaki et al., 2011). However, in large studies ALK rearrangements and EGFR activating mutations were mutual exclusive. In another study, amplification of KIT and increased autophosphorylation of EGFR was observed in 2/18 re-biopsies (Katayama et al., 2012). In some patients combinations of multiple resistance mechanisms were observed in the same tissue re-biopsy (Socinski et al., 2013). The data of these studies led to the concept that tumor cells can apply both ALK dependent and independent mechanisms to become resistant to crizotinib. In ALK gatekeeper mutant cases, the tumor cells remain addicted to ALK signaling. In contrast, ALK independent mechanisms refer to cases that have become addicted to an alternative oncogene (Doebele et al., 2012).

In clinical settings the majority of resistant mechanisms appear to be ALK dependent, since responses are seen in up to 70% of crizotinib resistant tumors on second line ALK inhibitors like ceritinib, alectinib and brigatinib (Table 1). These drugs have a higher potency thereby avoiding the possibility of bypassing the ALK signaling cascade via other pathways (Pall, 2015).

3.2. ALK inhibitor resistance in cell lines and xenograft mice models

3.2.1. Gatekeeper mutations

Resistance to crizotinib has been studied in several cell lines (Table 5). The H838 and H23 cell lines carrying wild type ALK gene alleles, have been used as a control for the ALK break-positive cell lines H3122, Ba/F3 and H2228 (Zhang et al., 2011). The SNU-2535 cell line carries besides an ALK break also a mutated ALK allele, i.e. G1269A (Kim et al., 2013b). The DFCI076 cell line gained a L1152R mutation (Sasaki et al., 2011). ALK gatekeeper mutations have been described as the major resistance mechanism in different cell lines treated with crizotinib. Resistant H3122 cells (H3122-CR) mainly gained C1156Y, F1174L, L1196M and G1269A gatekeeper mutations (Sasaki et al., 2011; Sang et al., 2013; Katayama et al., 2012; Kim et al., 2013b, 2013c; Huang et al., 2013; Tanizaki et al., 2012). The G1269A gatekeeper mutation carried by SNU-2535 and H2228 cells indeed showed increased resistance to crizotinib (Kim et al., 2013b).

The L1152R mutation present in the patient derived DFCI076 cell line also decreased sensitivity to crizotinib (Sasaki et al., 2011). Ba/F3 cells containing one of the three most effective resistance inducing gatekeeper mutations, i.e. L1196M, S1206R or G1269S, were insensitive to crizotinib in a xenograft model of SCID mice (Zhang et al., 2011). Introduction of the T1151K, L1152V, L1152R, C1156Y, I1171T, S1206R, E1210K, F1245C or G1269S gatekeeper mutations in the Ba/F3 cell line by site-directed mutagenesis, increased resistance to crizotinib by tenfold in each of the Ba/F3 subclones (Sasaki et al., 2011; Choi et al., 2010; Doebele et al., 2012; Zhang et al., 2011). So there is solid evidence that ALK gatekeeper mutations are involved in resistance towards crizotinib.

Table 4

Overview of re-biopsy studies in patients treated with crizotinib.

Re-biopsy study	N	Mechanism	mutations	Technique	ref
Sasaki et al. (2011)	1	ALK mutation	L1152R	DNA Sanger sequencing	Sasaki et al. (2011)
Sang et al. (2013)	1	ALK mutation	G1269A	DNA Sanger sequencing	
Katayama et al. (2012)	4/18	ALK mutation		DNA Sanger sequencing	Katayama et al. (2012)
	1/18	ALK gain		FISH	
	4/18	Higher levels of phospho-EGFR		IHC	
	2/18	KIT amplification		Snapshot multiplexed genotyping assay, FISH	
	1/18	Overexpression of KIT ligand stem cell factor (SCF)		IHC	
Kim et al. (2013)	3/7	ALK mutation	L1196M, G1269A	DNA Sanger sequencing	Kim et al. (2013b)
	1/7	ALK gain		FISH	
	1/7	EGFR activating mutation	L858R	DNA Sanger sequencing	
	1/7	EGFR polysomy		FISH	
	2/7	Overexpression of amphiregulin or EGF		ELISA	
Huang et al. (2013)	3/13	ALK mutation	G1269A, C1156Y, L1196M	DNA Ion Torrent sequencing	Huang et al. (2013)
Choi et al. (2010)	1		C1156Y, L1196M	RNA paired-end sequencing of ALK kinase domain	Choi et al. (2010)
Doebele et al. (2012)	4/11	ALK mutation	L1196M, G1269A	DNA direct sequencing	Doebele et al. (2012)
	2/11	ALK gain		FISH	
	2/11	Loss of ALK		FISH	
	1/11	EGFR activating mutation	L858R	DNA direct sequencing	
	2/11	KRAS mutation	G12C, G12V	DNA direct sequencing	
Jiang et al. (2013)	1	ALK mutation	G1548E	Illumina Hiseq 2000	Jiang et al. (2013)

Table 5Overview of IC₅₀ in cell lines used in research on crizotinib resistance.

Cell line	Alterations		Crizotinib(uM)	ref
	ALK	other		
H23	wt		1.7	Zhang et al. (2011)
H838	wt		1.3	Zhang et al. (2011)
H2228	ALK-EML4		0.01	Zhang et al. (2011), Kim et al. (2013c)
H2228-CR		EGFR ↑ BIM ↓	0.3	Kim et al. (2013c)
H3122	ALK-EML4		0.07–0.19	Sasaki et al. (2011), Sang et al. (2013), Katayama et al. (2012), Huang et al. (2013), Kim et al. (2013c), Tanizaki et al. (2012), Yamaguchi et al. (2014), Ji et al. (2014)
H3122-CR	Gatekeeper mutations ^a		0.24–0.26	Sasaki et al. (2011), Sang et al. (2013), Katayama et al. (2012), Huang et al. (2013), Kim et al. (2013c), Tanizaki et al. (2012), Yamaguchi et al., (2014), Ji et al. (2014)
H3122-CR	F1174L		0.62	Sasaki et al. (2011)
H3122-CR1		EMT	>3	Sang et al. (2013), Ji et al. (2014)
H3122-TR		EGFR ↑	2.56	Sasaki et al., (2011), Kim et al. (2013c)
DFCI076	ALK-EML4, L1152R		1.01	Sasaki et al. (2011)
Ba/F3	ALK-ELM4		0.02–0.07	Sasaki et al. (2011), Katayama et al. (2012), Choi et al. (2010), Doebele et al. (2012), Zhang et al. (2011)
Ba/F3	Gatekeeper mutations ^b		0.2–0.9	Sasaki et al. (2011), Katayama et al. (2012), Choi et al. (2010), Doebele et al. (2012), Zhang et al. (2011)
SNU-2535	ALK-EML4, G1269A		8	Kim et al. (2013b)

wt: wild type.

^a C1156Y, L1196M, G1269A, L1152R.^b L1196M, G1202R, S1206Y, G1269S, I1151Tins, T1151K, L1152V, L1152R, C1156Y, I1171T, E1210K, F1245C.

3.2.2. EGFR

Activation of the EGFR signaling pathway, as another mechanism of resistance to ALK inhibitors, was demonstrated in subclones of H3122 treated with crizotinib. Treatment of these H3122-CR cells with EGFR-TKI resulted in inhibition of cell growth (Tanizaki et al., 2012; Yamaguchi et al., 2014). Treatment of nude mice injected with H2228 NSCLC cells that overexpress the *EML4-ALK* fusion protein with crizotinib resulted in resistant xenografts that showed upregulation of the EGFR signaling pathway. These xenografts appeared to be sensitive to a combination of crizotinib and ganetespib. Ganetespib inhibits different HSP90 clients, including EGFR and may thus target the EGFR resistance associated activation in

these cells and explain the enhanced sensitivity to the combined treatment. HSP90 inhibitors also directly have impact on ALK stability and therefore they are used to treat ALK resistant patients. Functional loss of the signaling cascades was also associated with increased BIM protein expression. Based on these findings the authors suggested that BIM and EGFR upregulation, without activating mutations, is associated with crizotinib resistance (Sang et al., 2013).

3.2.3. Autophagy

Another mechanism proposed in H3122-CR cells was the induction of autophagy. Inhibition of the autophagy pathway by

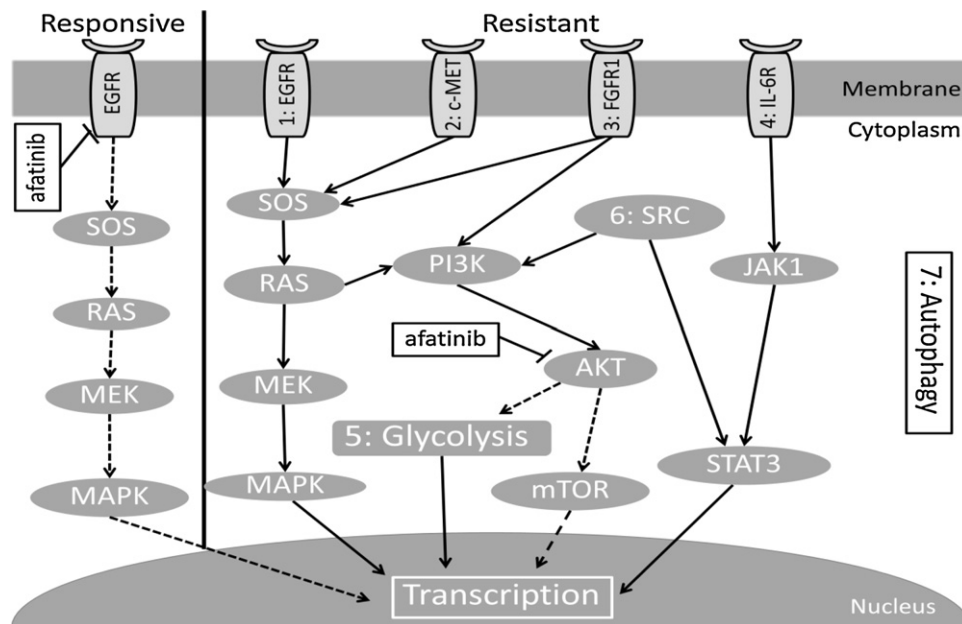


Fig. 1. Overview of probable resistance mechanisms in EGFR-TKI afatinib.

Legend: Proven resistance mechanisms include gatekeeper mutations (1) and MET amplification (2). The V843L mutation is a proven mechanism of resistance while this is most likely not the case for the T790M mutation. MET amplification is a proven resistance mechanism, but MET mutations probably not. All other proposed mechanisms (3–7) need to be studied in more detail to prove their causal resistance in patient samples. FGFR1 amplification (3), IL-6R/JAK1/STAT3 pathway (4) and autophagy (7) have been associated with resistance. The glycolysis pathway (5) induced by AKT has been proposed, although it is not clear why resistance occurs. Src upregulation (6) has been associated with resistance. Dashed arrow inhibited pathway, solid line arrow induced pathway.

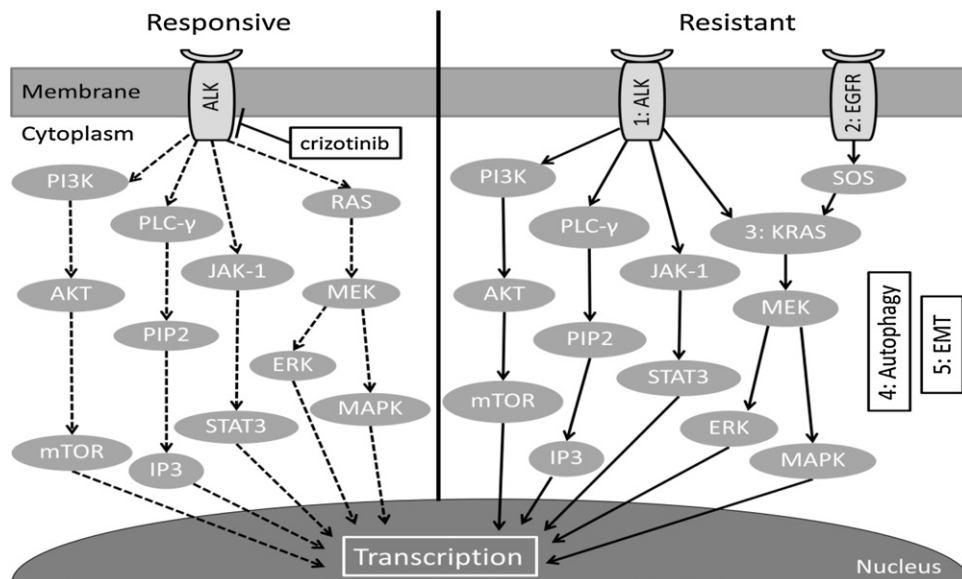


Fig. 2. Overview of possible resistance mechanism induced upon treatment with the ALK inhibitor crizotinib.

Legend: The only proven resistance mechanisms is gain of ALK gatekeeper mutations (1) due to lower binding efficiency of crizotinib. Other possible resistance mechanisms include EGFR (2) upregulation which are associated with resistance to crizotinib in cell lines and mutations, which have been found in patients resistant to crizotinib; KRAS mutations (3) have been found in patients treated with afatinib; autophagy (4) has been found in a crizotinib resistant cell line; epithelial-mesenchymal transition (5) has been found in resistant cell lines to crizotinib. Possible clinical relevance of these mechanisms are not clear yet. Dashed arrow inhibited pathway, solid line arrow induced pathway.

chloroquine and bafilomycin showed increased sensitivity to crizotinib (Ji et al., 2014). However, the clinical relevance of autophagy in the development of crizotinib resistance is not known.

3.2.4. Epithelial-mesenchymal transition (EMT)

Phenotypical alterations of the cell morphology due to down-regulation of E-cadherin and AXL and upregulation of vimentin are characteristics of EMT that have also been implicated in crizotinib

resistance. H2228 crizotinib resistant cells did not have secondary mutations, but did show epithelial-mesenchymal transition (EMT). EMT induced by TGF- β 1 revealed resistance to crizotinib in lung cancer cell lines that was reversible by removal of the TGF- β . Suppression of vimentin in H2228-CR cells by siRNA treatment restored sensitivity to crizotinib (Kim et al., 2013c). Thus, EMT may be a mechanism of resistance to crizotinib treatment as well.

4. Concluding remarks

Afatinib, erlotinib and gefitinib are the main drugs used to target EGFR and crizotinib is the main drug to target ALK. Alternative TKIs are still under investigation in clinical studies. For EGFR, the T790M mutation is important in the development of resistance to reversible TKI. The current literature is contradictory about its role in irreversible EGFR blocking agents such as afatinib. Clinical relevant concentrations for afatinib (0.08 μM) and dacomitinib (0.04 μM) are much lower than those for both erlotinib (4 μM) and gefitinib (0.9 μM) (Goss et al., 2005; Hidalgo et al., 2001; Bello et al., 2013; Yap et al., 2010). In relation to the effective dose required to inhibit cell growth of T790M positive lung cancer cell lines, it is unlikely that clinical effects will be achieved with the EGFR-TKIs in patients with a secondary T790M mutation. Similar, there is also no support for a role of *HER2* amplification, although this has been observed as a resistance mechanism after first generation TKI. This is probably due to the fact that afatinib is a *HER2* inhibitor as well. One of the most evident afatinib resistance mechanisms is amplification of *MET* (Fig. 1). Current studies indicate that resistance is not well associated with *MET* mutations or positive *MET* protein staining. The V843I mutation affecting binding of afatinib to EGFR causes resistance at least in cell lines. Overexpression of *FGFR1*, increased IL6R/JAK/STAT signaling, enhanced interference with the aerobic glycolysis, autophagy and SFK all have been associated with resistance to afatinib in one or more lung cancer cell lines. Until now, for afatinib the only proven resistance mechanisms include the V843I mutation and *MET* amplification (Fig. 1). Other mechanisms studied only in cell lines need to be confirmed in afatinib resistant tumor samples.

The main mechanism of crizotinib resistance is gain of a gatekeeper mutation leading to less effective binding of crizotinib to the mutated ALK kinase domain (Fig. 2). This has been shown in both patient samples and in cell lines. As only 40% of patients with clinical resistance have such gatekeeper mutations, there have to be other mechanisms in the remaining 60% of patients. Analysis of re-biopsies of patients upon crizotinib resistance revealed *ALK* amplifications, *EGFR* and *KRAS* mutations. The causal nature of these mutations needs to be confirmed and in a significant proportion of the patients the resistance mechanism remains unknown. Alternative mechanisms studied only in lung cancer cell lines include activation of the EGFR pathway, induction of the autophagy pathway and EMT. So, besides *ALK* gatekeeper mutations, all other proposed resistance mechanisms need to be studied in more detail to either proof the real cause of the observed resistance and whether these mechanisms also really determine resistance in patients treated with crizotinib *in vivo*.

4.1. Future perspectives

It is evident that in depth studies on the proposed resistance mechanisms are required to elucidate the full spectrum of TKI resistance. The discrepancy between clinical and cell line or xenograft results are due to the small therapeutic index of second generation EGFR TKI, such as afatinib. Clinical achievable doses of EGFR TKIs providing a balance between efficacy and side effects were too low to suppress T790M efficiently in EGFR mutant patients. Another known strategy to overcome resistance is to use a TKI drug holiday. During this drug holiday patients receive cytotoxic chemotherapy. After progression on this treatment, a re-challenge of TKIs can be given with the same TKI as used in first line, indicating that the initial resistance is not caused by mutations (Becker et al., 2011). Prospective studies are currently being performed to define response rates using this strategy. Beside this, next generation TKIs have been developed to overcome resistance. Those smaller molecules fit better in the functional tyrosine kinase pocket

than in the wild type pocket, e.g. osimertinib and rociletinib for EGFR mutation positive cases and ceritinib, alectinib, brigatinib and lorlatinib for *ALK* translocations (Jänne et al., 2015; Sequist et al., 2015; Pall, 2015; Mehra et al., 2012; Shaw et al., 2015). The renewed sensitivity towards TKIs after a drug holiday may support a role of epigenetic mechanisms, however these mechanisms have not been studied yet for the TKIs discussed here in lung cancer. A study with the HDAC inhibitor entinostat showed that a subgroup of patients with EMT had an advantage in OS. Therefore, further biomarker studies are needed to define which patients will respond to adding HDAC inhibitors (Witta et al., 2012). Resistance to EGFR-TKIs is associated with a *BIM* deletion polymorphism in cell lines. HDAC vorinostat restored the cell death pathway in cell lines with the *BIM* deletion and overcame resistance to gefitinib (Nakagawa et al., 2013). Moreover, HDAC inhibitors in combination with HSP90 inhibitors have a synergistic effect on inhibition of growth of TKI resistant cell lines (Sun et al., 2013b). This combination has not been tested in a clinical setting yet. Next generation sequencing of resistant tumor samples and functional studies of observed recurrent alterations can help to explain the complex pattern of resistance after exposure towards TKI. Current studies suggest involvement of multiple pathways as possible resistance mechanisms and these should be functionally tested in cell lines to prove a causal relation. Novel gene editing techniques such as CRISPR-cas and TALENS may speed up elucidation of specific resistance mechanisms in cell lines that can be validated in patient derived xenografts for the different EGFR-TKIs and ALK inhibitors. Efficacy studies using combination therapy to prevent resistance to TKI may be a promising strategy for future clinical trials. Moreover, new therapeutic strategies are available to overcome the currently known resistance mechanisms.

Conflict of interest

None.

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